Universidade de Aveiro
Ano 2009
Departamento de Biologia

Sónia Dias Coelho

Avaliação da toxicidade de Dipterex e Oxitetraciclina em Condições Tropicais

Toxicity Assessment of Dipterex and Oxytetracycline in Tropical Conditions

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Professor Doutor António Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação de Doutora Paula Inês Borralho Domingues, Bolseiro Pós-Doutoramento, Departamento de Biologia da Universidade de Aveiro

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palavras-chave Aquacultura, Tailândia, Nile tilapia, Peixe-Zebra, Químicos, Dipterex, Oxitetraciclina

resumo Alguns químicos são frequentemente usados em várias práticas da Aquacultura, principalmente no tratamento de peixes contaminados e para melhorar a qualidade da água. Na Tailândia, durante os últimos 20 anos, a indústria de cultivo intensivo expandiu consideravelmente tendo um papel importante na produção de produtos para exportação e no desenvolvimento sócio-económico do país. Consequentemente o uso de químicos tornou-se crescentemente uma parte da manutenção como forma de obter resultados rápidos. O dipterex e a oxitetraciclina são dois dos químicos mais usados no tratamento de peixes de água doce em ambiente tropical. Como estes podem acumular na água, solo e animais cultivados, causando impactos adversos no ambiente em geral, alguns cuidados devem ser tomados relativamente ao seu uso como forma de desenvolver boas práticas em sistemas de aquacultura. Para o alcançar, algumas medidas devem ser tomadas incluindo uma avaliação correcta da toxicidade dos químicos usados, o controlo da quantidade e qualidade dos produtos, melhoramento do tratamento de resíduos e monitorização das descargas de efluentes. O objectivo deste trabalho foi avaliar a toxicidade de dipterex e oxitetraciclina em condições tropicais para Nile tilapia e Peixe-Zebra. Nile tilapia (*Oreochromis niloticus*) é um dos peixes de água doce mais importantes cultivados na Tailândia. As larvas forem usadas para testar ambos os químicos e os valores de LC50 (96h) obtidos foram 3.96 mg/L e 1321.34 mg/L para dipterex e oxitetraciclina respectivamente. No entanto, estes valores não são definitivos uma vez que resultam de testes preliminares. O Peixe-Zebra (*Danio rerio*)é considerado como um bom modelo vertebrado. Neste trabalho foram usadas as fases de vida iniciais para testar o efeito tóxico do dipterex, e o valor de LC50 (96h) obtido foi 25.41 mg/L. Além disso mostrou ser teratogénico a elevadas concentrações.

Os químicos foram não tóxicos ou praticamente não tóxicos para os peixes de água doce estudados, no entanto este trabalho foi importante uma vez que não existia informação adequada acerca da toxicidade do dipterex e da oxitetracilina em ambiente tropical.

keywords Aquaculture, Thailand, Nile tilapia, Zebrafish, Chemicals, Dipterex, Oxytetracycline.

abstract Some chemicals are widely used in several Aquaculture practices, mostly to treat diseased fish and to improve water quality. In Thailand, during the last 20 years, the industry of aquaculture specially farming has expanded considerably playing an important role in the production of export products and in the socio- economic development of the country. Consequently the use of chemicals has become increasingly a part of management as a way to achieve quick results. Dipterex and oxytetracycline are two of the most used chemicals in the treatment of freshwater fish in tropical environment. As they can accumulate in water, soil and farmed animals, causing adverse impacts to the environment in general, attention has to be driven to their use in order to develop good management practices in aquaculture systems. To achieve this, several measures must be undertaken including an accurate evaluation of toxicity of the used chemicals, the control of the quantities and quality of the products, the improvement of the treatment of waste products and the monitoring of effluents discharges .The objective of this work was to evaluate the toxicity of dipterex and oxytetracycline in tropical conditions to Nile tilapia and Zebrafish. The Nile tilapia (*Oreochromis niloticus*) is one of the most important freshwater fish cultured in Thailand. Their larvae were used to test both chemicals and LC50 (96 hr) values of 3.96 mg/L and 1321.34 mg/L for Dipterex and oxytetracycline respectively were obtained. However, these results are not definitive as they provide from preliminary tests. Zebrafish (*Danio rerio*) is considered as a good vertebrate model. In this work early life-stages were used to test the toxic effects of Dipterex and a LC (96 hr) value of 25.41 mg/L was obtained. Moreover it showed to be teratogenic at high concentrations.

The chemicals were not highly toxic or practically non-toxic to the studied freshwater fish; however this work was important as no adequate information existed concerning dipterex and oxytetracycline toxicity in tropical environments.

“Science involves confronting our “absolute stupidity”.(…) The more comfortable we become with being stupid, the deeper we will wade into the unknown and the more likely we are to make big discoveries.”

Martin A. Schwartz (2008)

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1. General Introduction

1.1. Aquaculture in Thailand

Aquaculture is a worldwide tradition that is thought to be original from China with the development of early fish husbandry techniques about 4000 years ago. This knowledge was carried and passed on to neighbouring countries by emigrating Chinese; the practice extended into Thailand and throughout South East Asia, where aquaculture has been practiced for approximately 400 years (Stewart and Bhujel, 2007). It may have started as early as 1691, although for ornamental gold fish production rather than for food (Edwards and Virapat, 2005). The practice continued, with the production gradually rising throughout the 1900's (Stewart and Bhujel, 2007), in 1922 the Chinese immigrants imported fry Chinese carp by boat from the People's Republic of China (Edwards and Virapat, 2005) for culture in the region of Bangkok (Pongsri and Sukumasavin, 2005).

The Thai Department of Fisheries (DOF) set up an aquaculture promotion programme in 1951 (Pongsri and Sukumasavin, 2005). During the 1950's, DOF imported and spread Mozambique tilapia (*Oreochromis mossambicus*) that become a popular farmed fish in ponds, with technical assistance from the Food and Agriculture Organization (FAO) of the United Nations (Edwards and Virapat, 2005). Tilapia farming became established in 1965 following the introduction of Nile tilapia (*Oreochromis niloticus*) after His Majesty King Bhumipol received specimens as a present from His Imperial Highness Emperor Akihito of Japan when the second was Crown Prince (Edwards and Virapat, 2005).

More than 50 freshwater aquatic species have been cultured, at the present time and the five most important species, in terms of annual production, are Nile tilapia, hybrid catfish, silver barb, giant river prawn and snakeskin gourami (Pongsri and Sukumasavin, 2005; Stewart and Bhujel, 2007).

Three stages can be defined in the aquaculture development in Thailand. In the first stage the objective was to fulfil domestic food demand and to steady

social security by ensuring employment in rural areas. In the second stage, aquaculture gradually played an important role in producing commodities for export, as a result of technical developments and the government policy support to agricultural sectors and rural development. Presently, in the third stage, aquaculture is in a transition period aiming to be more harmonized with the natural environment and more consistent with socio-economic development (Tabthipwon, 2008). Thus, the total production increased over time. In 1950 the national total for aquaculture production in Thailand was only about 24,000 tons, a pointed increase was observed in 1960 followed by another increase in the late 80's and over the past 20 years an increment of 800% was verified (Figure 1.1). In 2004, aquaculture production in Thailand neared 1.2 million tons which has maintained the country's position as the 7th largest aquaculture producer in the world (Table 1.I) and lead Thailand to become a centre for aquaculture development/business (Stewart and Bhujel, 2007).

Table 1.I - Top ten aquaculture producers in 2004 (Source: (Stewart and Bhujel, 2007)

Country	Production volume	Global	Production value	Global
	Million tonnes	%	(Billion US$)	%
China	41.33	69.6	35.997	51.2
India	2.47	4.2	2.936	4.2
Philippines	1.72	2.9	0.794	1.1
Indonesia	1.47	2.5	2.163	3.1
Japan	1.26	2.1	4.242	6.0
Vietnam	1.23	2.1	2.459	3.5
Thailand	1.17	2.0	1.587	2.3
S. Korea	0.95	1.6	1.212	1.7
Bangladesh	0.91	1.5	1.363	1.9
Chile	0.69	1.2	2.815	4.0

The fisheries sector plays an important role in the national economy, foreign exchange earning, food production and employment for the rural population (Pongsri and Sukumasavin, 2005; Tabthipwon, 2008).

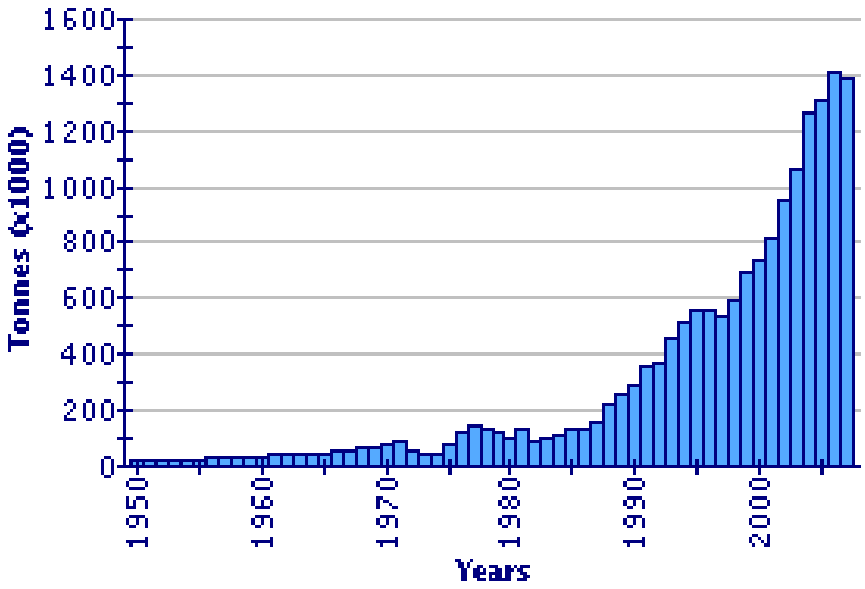


Figure 1.1- Reported aquaculture production in Thailand (from 1950) (Fao Fishery Statistic) Source: National Aquaculture Sector Overview Thailand

In 2003, total production reached 703,300 tones for coastal aquaculture and 361,125 tones for freshwater aquaculture (Tabthipwon, 2008).

The freshwater aquaculture includes culture in ponds, cages, paddy fields and ditches and most of the farms are located in areas rich in water resources or which are irrigated (Pongsri and Sukumasavin, 2005). In Thailand, in 2002 the number of fish local farms (most of these were pond farms) was 390853 which covered approximately 131500 ha. In 2004 the number of registered farms was over 440000 (Pongsri and Sukumasavin, 2005).

The production of Nile tilapia (*Oreochromis niloticus*) contributes approximately with 29% of total freshwater aquaculture production, followed by the walking catfish (Pongsri and Sukumasavin, 2005).

Like any practice that interacts with the surrounding ecosystems, as the aquaculture becomes more and more widespread and increases in intensity at international scale, it will generate an increasing environmental impact (Stewart and Bhujel, 2007).

1.2. Chemicals used in aquaculture and toxicology

In aquaculture, as in all food production sectors, chemicals are one of the most determinant external inputs for a successful production (Subasinghe et al., 1996). Thus, as a consequence of the development of Thai aquaculture, the use of chemicals has become increasingly a part of management, used mostly to treat diseased animals and to achieve a high water quality (Tonguthai, 1996). In the simple extensive systems the use of chemicals may be limited to fertilizers, while in complex semi-intensive and intensive systems an ample range of natural and synthetic compounds can be used (Subasinghe et al., 1996).

Numerous pathogens that cause diseases, in fish and shellfish, are facultative forms that are everywhere in aquatic systems. The diseases expand in aquaculture systems, usually, as a result of a disruption of the normal environment (Meyer, 1991). If the conditions are critical, such as temperature fluctuations, low levels of dissolved oxygen, crowding, excessive handling, inadequate diets and toxic substances, animals can become stressed and if the stress level exceeds the capacity to adjust, effect can be lethal (Meyer, 1991). So, chemicals are used to reduce transport stress and to control pathogens, among others applications, they increase production efficiency, as hatchery production and feeding efficiency and improve survival of fry and fingerlings to marketable size (Subasinghe et al., 1996).

There are some concerns with regard to the use of the chemicals such as human health concerns associated to the use of feed additives, therapeutants, disinfectants, hormones, and vaccines; product quality concerns; environmental concerns, such as the effects of aquaculture chemicals on water and sediment quality, natural aquatic communities, and effects on microorganisms; and the lack of knowledge concerning the effects and fates of chemicals and their residues in cultured organisms, in the aquaculture system itself and in the aquatic environment in general (Subasinghe et al., 1996). Human health and environmental concerns about the use of chemicals in aquaculture are reflected in the FAO Code of Conduct for Responsible Fisheries (Subasinghe et al., 1996).

The chemicals used in aquaculture have specific effects and can be applied singularly or in combination, however for their correct use, the water, the fish, the chemical and the disease characteristics must be known and considered (Tonguthai, 1996). One of the advantages for chemical use is that it achieves quick results (Tonguthai, 1996).

In Thailand, some of the chemicals are used discreetly in isolated cases, thus it is not possible to list all used in aquaculture, moreover some of the products are identified by their trade names with no further information on ingredients (Tonguthai, 1996).

1.2.1. Some Chemicals used in Aquaculture in Thailand

1.2.1.1. Chemicals typically used in Soil and water treatment (Tonguthai, 1996):

Lime is a chemical used to stabilize water pH, correct pond bottom, and also to guarantee healthy plankton normally

Teaseed Meal it is used in the ponds to kill predators and unwanted species before stocking, made up of ground teaseed with saponin.

Chlorine eliminates harmful organisms entering the pond with water.

Dolomite $CaMg(CO_3)_2$) used to afford Mg^{++} and to improve the buffering capacity.

Organic and inorganic fertilizers needed to fertilize the development of plankton that is very important as food for herbivorous fish like tilapia and milkfish.

Disodium Ethylene Diamine Tetraacetate (EDTA) reduces the heavy metal concentrations improving water quality.

1.2.1.2. Chemicals used as Chemotherapeutants (Tonguthai, 1996):

The **disinfectants** kill the bacteria and other pathogenic organisms. The substances usually used in Thailand as disinfectants include iodine, chlorine, benzalkonium choride (BKC) and formalin.

Iodine is used as a disinfectant in ponds at 1-5 gm/m^3 and hatcheries.

Chlorine is used to disinfect water in various forms, the most commons are chlorine gas (Cl_2), sodium hypochlorite (NaOCl) and calcium hypochlorite ($Ca(OCl)_2$).

Benzalkonium Chloride (BKC) one of the disinfectants used in Thailand to reduce the concentration of dinoflagellates and plankton on closed pond systems.

Formalin, besides being used as a disinfectant it has been used as treatment against external parasites (ciliated proypzoans and monogeneas) in fish mainly in freshwater fish.

1.2.1.3. Chemicals frequently used as Therapeutants (Tonguthai, 1996)

Acriflavin is used in the treatment of fish eggs and aquarium fish in case of infections by bacteria and external protozoans.

Copper compound (Cutrine Plus) is one of the oldest and most used chemicals in fish culture; it is used as a parasiticide against infestations by external protozoans.

Dipterex (Dyxlon, Chlorofos, Masoten, Foschlor, Neguvon, Trichlorfon) is used to treat for monogenean, crustacean and protozoan parasites in pond-cultured fish.

Malachite green, a mixture of formalin and malachite green at a ratio of 25:0.1 ppm is used to treat “Ich” (*Ichthyophthirius multifilis*) in aquarium and pond fish.

Trifuralin (Treflan) is used as a prophylactic chemical against fungal infection in shrimp hatcheries.

Potassium permanganate ($KMnO_4$), it was one of the first chemicals used as a chemotherapeutant in aquaculture to treat infections provoked by external parasites such as monogeneans.

Salt (sodium chloride) is usually used in recirculating systems, it is useful in reducing some infections in a system and helps to reduce osmorality

stress rising the salt concentration in the water relative to the normal concentration in the fish's body (Yanong, 2003a).

1.2.1.4. Antibiotics (Tonguthai, 1996)

The major disease problems in freshwater and brackishwater aquaculture are the infections provoked by bacteria. In Thailand, the main problems in the culture of freshwater fish and other animals are caused by *Aeromonas hydrophila*.

Antibiotics are usually applied orally by mixing with feed or injection practical to large fish. Almost all the antibiotics used in aquaculture are medicines for human use too.

Erythromycin, used to treat vibriosis in shrimp larvae and bacterial infection in walking fish.

Oxytetracycline is approved by the US FDA (Food and Drug Administration) and has been widely used for a long time in the treatment of freshwater fish, frogs and softshell turtles, so its use might result in severe environmental contamination and has aroused concerns about its public health impacts. It is also used in the control of vibriosis and columnaris disease in fresh and brackishwater fish.

Nitrofurans (Furacin, Furanace), this group of antibiotics is considered dangerous for the humans because they have potential carcinogenic effects, though some of these have being extensively used in aquaculture. Furacin is used in the treatment of infection by *Vibrio* spp. in shrimp larvae.

Oxolinic acid is also approved by the US FDA for use in aquaculture and is used in shrimp treatment of vibriosis and in freshwater fish against *Aeromonas hydrophila*.

Sulphamonomethoxine (Dimeton) is used in the treatment of bacteria in fish.

1.2.1.5. Food additives (Tonguthai, 1996):

Besides the protein, carbohydrate and fat, the artificial food contain some additives such as vitamins, minerals, carotenoid pigments, phospholipids and others, to increase growth and survival of cultured animals.

Antibiotics are added to the artificial feed as growth promoters.

Hormones as corticosteroids, anabolic steroids and other steroids were integrated in feed in shrimp hatcheries to obtain larvae with healthy look and uniform size.

Vitamins; in extensive culture systems the natural food might be abundant enough to supply essential vitamins, however in intensive aquaculture systems in Thailand, the natural food is restricted, so the addition of vitamins to the diet is recommended. Vitamin C is extensively used in shrimp diets.

1.2.1.6. Immunostimulants (Tonguthai, 1996):

The overuse of chemicals in aquaculture is responsible for resistance development in some strains of pathogens, they become resistant to certain antibiotics, as the antibiotics do not totally eliminate the pathogens and residues may accumulate in fish flesh and the environment. Some scientists consider that the use of immunostimulants against infectious diseases is more beneficial than using antibiotics because there is no residue in tissues and strain resistance of bacteria to antibiotics is avoided.

Glucan, stimulates the non-specific defence mechanism of aquatic animals.

Peptidoglycan increases, significantly, the growth rate and survival of treated animals.

1.2.1.7. Vaccines (Tonguthai, 1996):

The injection and oral feeding are recommended for broodstock (sexually mature individuals) and pond grow-out while the immersion is recommended for

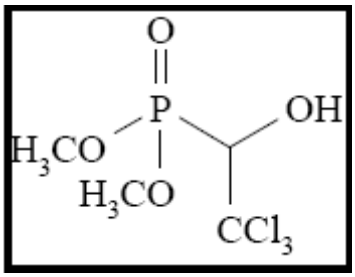
larvae. One of the vaccines used for fish in Thailand is the *Vibrio anguillarum* vaccine.

The pathogens can be transmitted by different ways: in the water, fish to fish, by vectors (organisms that can transmit other organisms capable of causing disease to another animal), by fomites (inanimate objects that can transmit diseases) and the food (Yanong, 2003a) Thus, it is necessary an adequate control of pathogens in the system which requires an understanding of where pathogens can be found, how they are transmitted to fish and how the numbers can be reduced. Also, understanding the appropriate chemicals to use to reduce or eliminate pathogens is a crucial part of good management (Yanong, 2003a).

1.2.2. Dipterex/ Trichlorfon

Organophosphates have been widely used as a relevant treatment for various parasitic infestations. Among those, the Trichlorfon appears with some frequency in literature in some works testing the after treatment effects in several species (Ranzani-Paiva et al., 1997).

The chemical name of the trichlorfon (dipterex) is O,O-dimethyl-(1-hydroxy-2,2,2-trichloroethyl)-phosphonate (Rodrigues et al., 2001), with the empirical formula $C_4H_8O_4Cl_3P$ (EPA, 1997).



It is an organophosphate insecticide (Anon., 1996) which acts as an acetylcholinesterase inhibitor (EPA, 1997) and has different names as Dylox, Dipterex, Proxol and Neguvon (EPA, 1997).

The trichlorfon is classified as a General Use Pesticide (GUP) by the U.S. Environmental Protection Agency (EPA) and it is in moderately toxic-toxicity class II (Anon., 1996).

Organophosphates are widely used to control agricultural pests and in the general treatment of ectoparasites in animals. In Aquaculture they are also used to control ectoparasites (Rodrigues et al., 2001).

The trichlorfon is used to control many insects as cockroaches, crickets, silverfish, bedbugs, fleas, cattle grubs, flies, ticks, leafminers, and leaf-hoppers, it is applied to vegetables, fruits, and field crops, ornamental and forestry plantings, in agricultural and domestic settings; in greenhouses, and for control of parasites of fish in selected aquatic environments, it is also used for treating domestic animals for control of internal parasites (Anon., 1996).

This organophosphate insecticide is a selective insecticide, meaning that it is specialized in certain insects, but spares many or most other organisms (Anon., 1996). It is a white crystalline solid with a melting point of 75-84º C and it is soluble in water, dichloromethane, 2- propanol, and toluene, and nearly insoluble in n-hexane (EPA, 1997) with percent active ingredient ranging from 40% to 98% (Anon., 1996).

The dipterex is highly toxic to many aquatic species, in both technical and formulated forms, for example Daphnia, stoneflies, crayfish, and several freshwater fish species (Anon., 2006) (Table 1.II). Normally, toxicity increases (i.e., observed LC50 is lower) with higher pH and higher temperature. There are no studies showing a potential for trichlorfon to accumulate in fish (Anon., 2006).

Table 1.II - LC50 (96hrs) of Dipterex in different fish

Fish	Dipterex LC50-96 hrs (mg/L)
Rainbow trout	1,4 (Anon., 1996)
Brook trout	2,5 (Anon., 1996)
Channel catfish	0,88 (Anon., 1996)
Bluegill	0,26 (Anon., 1996)
Nile tilapia	1,43 (Tuan, 1992)

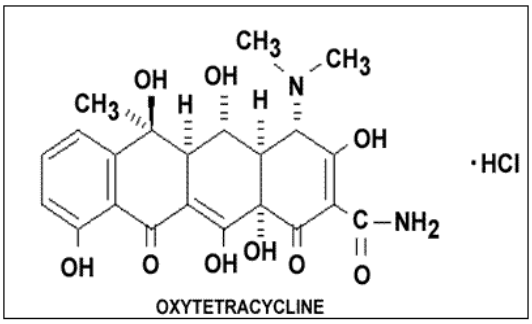
The dipterex breaks down quickly in aerobic soils, with a half-life between 3 to 27 days with an average half-life of 10 days, dichlorvos (DDVP) is the major breakdown product (Anon., 1996). It does not adsorb strongly to soil particles, so it is of low persistence in soil environments, is readily soluble in water, and very mobile in soils of varying textures and organic contents. It is consequently likely to contaminate groundwater (Anon., 1996). It degrades rapidly in alkaline pond water (pH 8.5) at room temperature, approximately 99% of applied trichlorfon degraded in 2 hours, but it remains stable in the same pond water kept under acidic (pH 5.0) conditions for 2 hours. The major breakdown product of trichlorfon in both soil and pond water is dichlorvos (DDVP, dimethyl 2,2-dichlorovinyl phosphate) (EPA, 1984; Anon., 1996). This insecticide was revealed to persist at detectable levels for 526 days in water at 20º C (Anon., 1996).

The insecticides such as dipterex have been extensively used and the amount applied by aquaculturists has increased considerably causing a big concern for the environment (Tonguthai, 1996).

1.2.3. Oxytetracycline

Oxytetracycline (OTC) is one widely used antibiotic in aquaculture and can be also added to fish and prawn food as a growth promoter (Choo, 1994).

The various forms of this antibiotic are oxytetracycline, oxytetracycline calcium complex, oxytetracycline hydrochloride (EPA, 1988). The chemical name is 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydro-6-methyl-1,ll-dioxo-2-naphthacenecarboxamide and its calcium complex and hydrochloride salts, and with the molecular formula $C_{22}H_{24}N_2O_9$ (EPA, 1988; O'Neil et al., 2001), $C_{22}H_{22}N_2O_9$ Ca (calcium complex) and $C_{22}H_{24}N_20_9$ HCL (hydrochloride) (EPA, 1988).



OXYTETRACYCLINE

OTC can be used as a pesticide, like a plant fungicide, bactericide and algicide or to control bacterial and fungal diseases and slime forming microorganisms (EPA, 1988). Some examples of use are: treatment of bacterial hemorrhagic septicemia and pseudomonas disease in catfish and salmonids, control ulcer disease, furunculosis, in salmonids and the bacterial disease gaffkemia in lobster (MacMillan et al., 2004).

In ornamental aquaculture, the OTC and related antibiotics are considered as common used antibiotics that work well when mixed with food against a wide variety of bacteria. The bath treatments may not be as effective for all species since the efficacy of OTC varies with the water hardness, increasing the water hardness (increase of calcium and magnesium levels) the dosage of these drugs in bath treatment has to increase necessarily Tetracyclines are unproductive if used as a bath treatment for saltwater fish (Yanong, 2003b).

Table 1.III - Oxytetracycline LC50 (96hrs) in different fish

Fish	OTC LC50- 96 hrs (mg/L)
Bluegill	>100 (Murphy and Peters, 1991b)
Striped bass	178 (Vaituzis, 1988)
Rainbow trout	> 116 (Murphy and Peters, 1991a)

Tetracyclines are light sensitive, and when they decompose they turn brown, contributing to poor water quality and may be dangerous to the fish (Yanong, 2003b), see on the Table 1.III some LC50 values.

The OTC degradation in seawater is light and temperature dependent and in aqueous solution is pH dependent, the half life of oxytetracycline hydrochloride in freshwater was determined as 58h (average pH 7.3. temperature 27ºC, under natural light) and 298h in seawater (average pH 7.9, temperature 27ºC , under natural light) (Choo, 1994).

In another work (Pouliquen et al., 2006), the degradation of the OTC was tested. They concluded that it was degraded by hydrolysis and photolysis after 14 days of exposure, about 70% of OTC were photodecomposed and about 20% were hydrolysed in freshwater and seawater; and that OTC hydrolysis and photolysis in freshwater and seawater were higher than in deionised water. They showed that OTC photolysis and hydrolysis increased when water pH increased, so water pH also played an important role in the OTC photolysis. Thus in water solutions the acidic conditions are ideal for OTC stability and alkaline conditions increased the OTC degradation rate (Pouliquen et al., 2006).

As the OTC has been used in aquaculture for a long time, it may have caused environmental contamination which consequently arises concerns about impacts on public health (Tonguthai, 1996).

1.3. Characterization of the species

1.3.1. Nile tilapia (*Oreochromis niloticus*)

The Nile tilapia (*Oreochromis niloticus*) was one of the first fish species being cultured, as it can be demonstrated in some illustrations from the Egyptian tombs, suggesting that the specie is cultured since 4000 years ago (Popma and Masser, 1999; Rakocy, 2006). It is naturally distributed in Palestine, in the Nile River as well as in most of African rivers and lakes (Bocek, 2008) and is still the most widely cultured specie of tilapia in Africa (Popma and Masser, 1999).

Nile tilapia (*Oreochromis niloticus,*Linnaeus, 1758) is a member of the family Cichlidae (Bocek, 2008). This specie belongs to the group of cichlids, which has the name "Tilapia" and consists in three aquaculturally important genera: *Oreochromis*, *Sarotheradon* and *Tilapia* (Popma and Masser, 1999; Bocek, 2008).

The *Tilapia* species are nest builders, the fertilized eggs are protected in the nest by a brood parent (Popma and Masser, 1999). The species from both

Oreochromis and *Sarotheradon* genera are mouth brooders, the eggs are fertilized in the nest and the parents instantly pick up the eggs in their mouths and hold them through incubation and for several days after hatching (Popma and Masser, 1999). Nile tilapia belongs to genus *Oreochromis* (Bocek, 2008). In this genus only the female is involved in the brood care, immediately after fertilization by the male, the female collects the eggs, incubates them into her mouth and moves off from the nest (Rakocy, 2006). Fry brooded up until free swimming (Bocek, 2008) and the yolk sac is absorbed (Rakocy, 2006). Depending in temperature, incubating and brooding is accomplished in 1 to 2 weeks, after fry are released, they may swim back into the female mouth if danger threatens (Rakocy, 2006). The egg number is proportional to the female's body weight (Rakocy, 2006).

Tilapia are shaped much like sunfish or crappie but are easily identified by an interrupted lateral line typical of the Cichlid family of fishes (Popma and Masser, 1999; Bocek, 2008). These animals have a laterally compressed body with long dorsal fins (Popma and Masser, 1999; Bocek, 2008), with 16-17

spines and 11 to 15 soft rays (Rakocy, 2006). Spines are also found in the pelvis and anal fins (Popma and Masser, 1999; Bocek, 2008) with 3 spines and 10 to 11 rays (Rakocy, 2006).

The mature Nile tilapia male have gray or pink pigmentation in the throat region (Popma and Masser, 1999). In the spawning season colour of the pectoral, dorsal and caudal fins become reddish (Rakocy, 2006).

The tilapia sexual maturity depends on the age, size and environmental conditions (Popma and Masser, 1999).

The fish farms use some strategies, that prevent the overcrowding and stunting, like: the cage farming where the eggs fall through the mesh to the pond floor before the female can collect them for brood; polyculture with a predator fish such as fingerling largemouth bass; and the monosex male culture (Popma and Masser, 1999). The use of male monosex populations is also desirable in ponds because the male tilapia grow approximately twice as fast as females (Popma and Masser, 1999; Rakocy, 2006), the males in general weigh from 600-800 gms in four months and can even grow more, and the females reach only as much as 150 gms in the same period (Bocek, 2008). For obtaining these populations the sex- reversal of female fry is one of the mainly used methods (Rakocy, 2006) Tilapia become sexually differentiated for several days after yolk sac absorption (Rakocy, 2006) so the hatched fry for 3 to 4 weeks are fed with a male hormone- treated feed (17 α methyltestosterone, MT) and they develop as phenotypic males. The percentage of reversal when fed with this hormone is 95-99% (Rakocy, 2006).

After the sex reversal the fingerlings are nursed to an advanced size before they are stocked into grow-out facilities to increase survival in the grow-out stage (Rakocy, 2006). See Nile tilapia production cycle on the Figure 1.2.

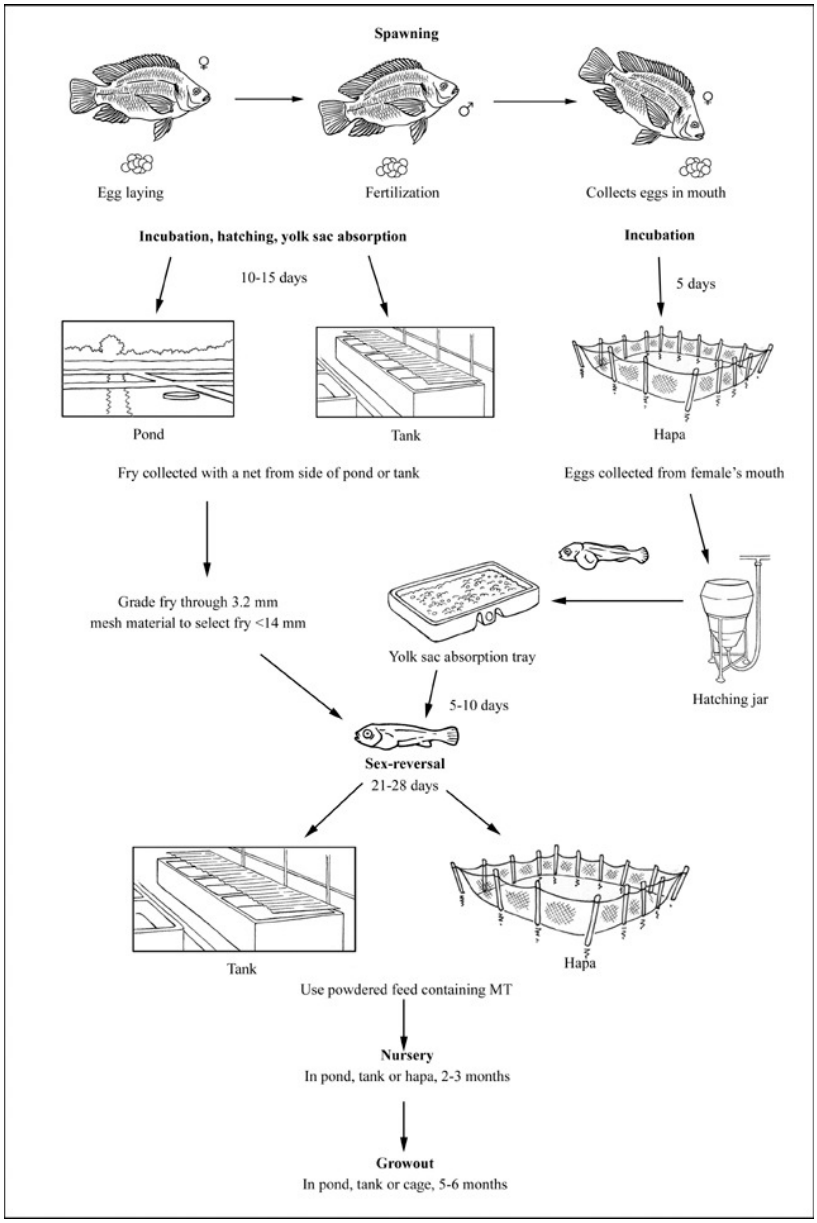


Figure 1.2 - Nile tilapia production cycle . Source: (Rakocy, 2006)

Tilapia eats a wide variety of natural food organisms, as well as, some aquatic macrophytes, planktonic and benthic aquatic invertebrates, plankton, larval fish, detritus and decomposing organic matter (Popma and Masser, 1999). The natural food organisms normally account for 30 to 50 percent of the tilapia growth, with heavy supplemental feeding (Popma and Masser, 1999), that increase available food for fish and are useful for fattening (Bocek, 2008). Prepared feeds that supply a complete diet (adequate protein, lipids, carbohydrates, vitamins and minerals) are available in developed countries and

are also manufactured and available in developing countries with an export market for elevated quality tilapia products (Rakocy, 2006).

As they are able to crop plankton from the water, tilapias are considered filter feeders, the gills secrete a mucous that traps plankton (Popma and Masser, 1999). In general tilapias consume natural food so efficiently that crops of more than 3,000 Kg/ha fish can be sustained in well fertilized ponds without supplemental feed, the nutritional value that this food provide in ponds is important, even for commercial operations that feed fish intensively (Popma and Masser, 1999). These fish are more tolerant than the most usually farmed freshwater to high salinity, high water temperature, low dissolved oxygen and high ammonia concentrations (Popma and Masser, 1999; Bocek, 2008).

The *Nile tilapia* is the least saline tolerant of the commercially significant species, but grows healthy at salinities up to 15ppt, they can reproduce in salinities of 10 to 15 ppt but perform better at salinities below 5 ppt. Fry numbers decline significantly at 10ppt salinity (Popma and Masser, 1999).

Relatively to the water temperature, the intolerance of tilapia to low temperatures is a serious restriction for commercial culture in temperate regions. Tilapias stop feeding when water temperature falls under 17.2ºC. Optimal water temperature for tilapia growth is about 28.3ºC to 31.1ºC and the reproduction is best at temperatures higher than 26.2ºC and does not occur under 20.0ºC (Popma and Masser, 1999).

The lack of oxygen results in poor growth and occurrence of diseases or mortality in fish. Usually, most warm water species require dissolved oxygen (DO) at a level of 1 ppm for survival and about 3 ppm for comfort. The most ideal DO concentration for growth and excellent fish health is 5 ppm, though, tilapia can grow healthy at DO level of 1 to 3 ppm (Bocek, 2008).

The ideal pH range of freshwater culture is 6.5 to 9.0. (Bocek, 2008), however, tilapia can survive in pH ranging from 5 to 10 (Popma and Masser, 1999).

Ammonia is highly toxic to fish (Bocek, 2008), tilapia substantial mortality occurs in a few days when fish are rapidly transferred to water with unionized ammonia concentrations superior than 2 mg/L. However, when gradually acclimated to sublethal levels, approximately half of the fish will survive 3 or 4

days at unionized ammonia concentrations as high as 3 mg/L (Popma and Masser, 1999).

At the optimum environmental requirements tilapia are more resistant to viral, bacterial and parasitic diseases, like the ones shown in the Table 1.IV, than the other usually cultured fish (Popma and Masser, 1999). The diseases can be avoided by maintaining a high quality environment and reducing handling stress (Rakocy, 2006).

Table 1.IV - The major disease problems affecting *Nile tilapia.*

DISEASE	AGENT	TYPE	SYNDROME	MEASURES
Motile Aeromonas Septicaemia (MAS)	*Aeromonas hydrophila* & related species	Bacteria	Loss of equilibrium; lethargic swimming; gasping at surface; haemorrhaged or inflamed fins & skin; bulging eyes; opaque corneas; swollen abdomen containing cloudy or bloody fluid; chronic with low daily mortality	$KMnO_4$ at 2-4 mg/litre indefinite immersion or 4-10 mg/litre for 1 hour; antibiotics (need 'extra-label use permit' in the USA), e.g. Terramycin® in feed at 50 mg/kg fish/d for 12-14 d, 21 d withdrawal
Vibriosis	*Vibrio anguillarum* & other species	Bacteria	Same as MAS; caused by stress & poor water quality	Antibiotic in feed
Columnaris	*Flavobacterium columnare*	Bacterium	Frayed fins &/or irregular whitish to grey patches on skin &/or fins; pale, necrotic lesions on gills	$KMnO_4$ as with MAS; indefinite immersion with $CuSO_4$ at 0.5-3 mg/litre, depending on alkalinity
Edwardsiellosis	*Edwardsiella tarda*	Bacterium	Few external symptoms; bloody fluid in body cavity; pale, mottled liver; swollen, dark red spleen; swollen, soft kidney	Antibiotic in feed
Streptococcosis	*Streptococcus iniae* & *Enterococcus* sp.	Bacteria	Lethargic, erratic swimming; dark skin pigmentation; exophthalmia with opacity & haemorrhage in eye; abdominal distension; diffused haemorrhaging in operculum, around mouth, anus & base of fins; enlarged, nearly black spleen; high mortality.	Antibiotic in feed, e.g. Erythromycin at 50 mg/kg fish/d for 12 d (requires 'extra-label use' permit in the USA)
Saprolegniosis	*Saprolegnia parasitica*	Fungus	Lethargic swimming; white, grey or brown colonies that resemble tufts of cotton; open lesions in muscle	$KMnO_4$ or $CuSO_4$ treatments; use 1 mg/litre of $CuSO_4$ for every 100 mg/litre alkalinity up to 3.0 mg/litre $CuSO_4$; formalin at 25 mg/litre indefinite immersion or 150 mg/litre for 1 h
Ciliates	*Ichthyophthirius multifiliis*; *Trichodina* & others	Protozoan parasite	Occurs on gills or skin	$KMnO_4$, $CuSO_4$ or formalin treatments
Monogenetic trematodes	*Dactylogyrus* spp.; *Gyrodactylus* spp.	Protozoan parasite	Occurs on body surface, fins or gills	Same as for ciliates

Source: Cultured Aquatic Species Information Programme, *Oreochromis niloticus* (Linnaeus, 1758).

Under good growth conditions, 1gram fish are cultured in nursery ponds to 20 to 40grams in 5 to 8 weeks and then restocked into grow- out ponds. With good temperature regimes, in monosex grow- out ponds, males normally reach

a weight of more than 200grams in 3 to 4 months, more than 400grams in 5 to 6 months and 700grams in 8 to 9 months. To the production of 400 to 500gram fish, the frequent practice is to stock 6000 to 8000 males per acre in static water ponds with aeration or 20000 to 28000 males per acre where 20% of the water is daily exchange. After 6 months feeding with good quality feeds such ponds can produce 2268 to 3175 Kg per acre and 8171 to 9072 Kg per acre, respectively (Popma and Masser, 1999).

Tilapia is known because of their rigidity, is very resistant to diseases, is ease breeding, has a reasonable growth rate, has a good taste and is very tolerant to a range of environmental conditions including temperature and salinity (Stewart and Bhujel, 2007; Bocek, 2008), therefore it has become the main farmed species produced for consumption within Thailand. Moreover, the government is playing an important role in promoting tilapia to the people as a cheap source of protein (Stewart and Bhujel, 2007).

This scale of mass production does not come without its cost to the natural environment (Stewart and Bhujel, 2007).

1.3.2. Zebrafish (*Danio rerio*)

Danio rerio is a freshwater fish which belongs to the family of freshwater fishes Cyprinidae and subfamily Rasborinae (Spence et al., 2007). This teleost Standard Length is 40 mm, the body shape is fusiform and is laterally compressed with a terminal oblique mouth directed upwards. The characteristics of the species are an incomplete lateral line extending to the pelvic fin base, two pairs of barbels and five to seven dark blue longitudinal stripes extending from behind the operculum into the caudal fin (Spence et al., 2007).

Males and females have similar colouration, although males tend to have larger anal fins with more yellow colouration, and gravid females have a more rounded body shape (Spence et al., 2007).

The natural origin of the zebrafish is around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal. There are approximately 44 danionin species throughout South and Southeast Asia,

their main species diversity in north-eastern India, Bangladesh and Myanmar (Spence et al., 2007). It can be found in inhabits streams, canals, ditches, ponds, beels and in slow-moving to stagnant standing water bodies, particularly rice-fields (Anon., 2009). The name *Danio* derives from the Bengali name "*dhani",* meaning "of the rice field" (Spence et al., 2007).

The zebrafish can survive in a wide range of temperatures, from as low as 6ºC in winter to over 38ºC in summer. It is omnivorous, the natural diet consists primarily of zooplankton and insects, although other contents have been reported from gut as phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales, arachnids, detritus, sand and mud (Spence et al., 2007).

1.3.2.1. Features of the zebrafish as a model animal system

Zebrafish has been a famous model vertebrate in a diversity of biological disciplines (Hill et al., 2005) because it has a number of characteristics which make it attractive as a model laboratory animal for developmental, toxicological and transgenic research (Lele and Krone, 1996). One of the significant advantageous features is the relatively low space requirements and maintenance costs of a zebrafish facility following the initial setup investment. A colony can be maintained in a fraction of the space which would be required for a comparable colony of larger fish models (Lele and Krone, 1996).

Since single embryos, it can be maintained in liquid volumes as small as 100 µl for the first five to six days of development, they can be kept in individual microtiter wells(Anon., 2009). In contrast to larger species, the small size of the larval and adult zebrafish is also favourable to minimizes costs because they require small quantities of dosing solutions (experimental chemicals, drugs and pollutants) leading to limited volumes of waste for discarding and minimizing quantities of labware and chemicals (Hill et al., 2005).

Females can spawn every 2-3 days and a single clutch may contain several hundred eggs (Spence et al., 2007), one pair of adult fish is capable of laying 200- 300 egg in one morning (Hill et al., 2005). Several pairs can be rotated to afford thousands of eggs daily and all year round, this can be

maximized by using newly matured fish that are between 3 and 6 months old (Hill et al., 2005).

Seven periods of embryogenesis of the *Danio rerio* have been defined (Kimmel et al., 1995): the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching, occurring during the first 3 days after fertilization Table 1.V.

Table 1.V - Embryogenesis period (according to Kimmel et al., 1995)

Period	H	Description
Zygote	0	The newly fertilized egg through the completion of the first zygotic cell cycle.
Cleavage	3/4	Cell cycles 2 through 7 occur rapidlyand synchronously.
Blastula	2 1/4	Rapid, metasynchronous cell cycles (8, 9) give way to lengthened, asynchronous ones at the midblastula transition; epiboly then begins.
Gastrula	5 1/4	Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly.
Segmentation	10	Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears.
Pharyngula	24	Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and tins begin development.
Hatching	48	Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously
Early larva	72	Swim bladder inflates; food- seeking and active avoidance behaviors.

Zebrafish completes embryogenesis in the first 72 hours and most of the internal organs, including the cardiovascular system, gut, liver and kidney develop rapidly in the first 24-48 hours. The embryos are completely transparent, facilitating observation and analysis. All the precursor tissues of the brain, eyes, heart and musculature can be easily visualized using light microscopy (Anon., 2009). Larvae display food seeking and active avoidance

behaviours within five days post fertilisation, i.e. 2 to 3 days after hatching (Spence et al., 2007).

Table 1.VI - Features of the zebrafish as a model laboratory animal (according to Lele and Krone, 1996)

Advantages	Disadvantages
low cost maintenance	lack of extensive veterinary knowledge
low space requirement on a per animal basis	lack of certain molecular techniques (e.g. gene knockout technology has not yet been developed)
rapid generation cycle (egg to mature adult in 2-3 months)	evolutionary position (primarily a problem for some forms of medically-related research)
large number of offspring	
well characterized developmental staging series	
rapid development (egg to hatching in 2-3 days)	
embryos well suited for experimental manipulalion and microinjection	
translucent embryos	

These features, Table 1.VI, make the zebrafish underexploited for organismal research, comprising studies of ecology, evolution and behaviour (Engeszer et al., 2007).

1.4. Aims and structure of the thesis

The sustainable and long term growth of the aquaculture industry causes an increase of the use of chemicals in farming operations; they can accumulate in the farmed animals, water and soil acting like environmental contaminants and putting fish health and quality in risk (Focardi et al., 2005). For this reason the principal objective was to test the toxic potential of two chemicals (Dipterex and Oxytetracycline) frequently used in aquaculture practices on tropical

environment, especially in Thailand like for example in the Nam Sai Farms Co. Ltd.

The toxicity potencial was tested in two freshwater fish species Nile tilapia (*Oreochromis niloticus*) and Zebrafish (*Danio rerio*) applying early-life stage acute toxicity tests and evaluating the mortality of the Nile tilapia and the mortality and response of the Zebrafish quantifying some endpoints.

Evaluating the toxicity of these chemicals in tropical environment it will enable to help in the decision about the correct concentrations to use in each aquaculture practice, like water and fish treatments, according to this environment.

This thesis is organized by 3 chapters.

(Chapter 1), General Introduction, this chapter that compiles information considered relevant for the understanding the developed work.

Chapter 2, "Toxicity assessment of two chemicals used in aquaculture: Dipterex and Oxytetracycline", describes how did Dipterex and Oxytetracycline affect the mortality and embryogenesis of Nile tilapia and Zebrafish.

Chapter 3, Conclusions and Final remarks.

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2. Toxicity assessment of two chemicals used in aquaculture: dipterex and oxytetracycline.

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Abstract

Aquaculture plays an increasingly important role in food security and in the economy in Thailand with a very high portion of total export products and national income. Technology of intensive farming has expanded considerably in the last 20 years. With this long-term growth of the aquaculture industry a wide range of environmental contaminants are discharged into the environment, such as the chemicals used in farming operations, like Dipterex and Oxytetracycline widely used in the treatment of freshwater fish. These chemicals can accumulate in farmed animals, water and soil and put fish health and quality at risk. As the chemicals and drugs will continue to play an important role in the development of Thai aquaculture it is necessary to develop good management practices for aquaculture systems, and consequently, to apply some toxicological analysis to the chemicals used in aquaculture operations. Thus the objective of this work was to evaluate the toxicity of dipterex and oxytetracycline in tropical conditions to Nile tilapia and Zebrafish.

One of the most important freshwater cultured species is the Nile tilapia (*Oreochromis niloticus*), and it was used to test the dipeterex and oxytetracycline toxicity, the larvae were used in Acute Toxicity Tests to obtain the LC50 (96 hr) values of 3.96 mg/L and 1321.34 mg/L respectively. Zebrafish (*Danio rerio*) was used to test the dipterex toxicity by early life stages tests, the LC50 (96 hr) obtained value was 25.41 mg/L, it showed to be teratogenic at high concentrations.

Althought the chemicals showed to be not highly toxic or practically non-toxic to the studied fish, this work was important to test the effects because no adequate data and information existed in support of the toxicity of these chemicals in tropical environments.

Key words: Aquaculture, Thailand, Tilapia, Zebrafish, Chemicals, Dipterex, Oxytetracycline.

2.1. Introduction

Aquaculture in Thailand plays a crucial role in food security, national economy (Pongsri and Sukumasavin, 2005) and employment for the rural population, with a very high contribution to the total export products and national income (Tabthipwon, 2008). In the last two decades this practice and its technology of intensive farming has expended considerably with freshwater and marine farming (Tabthipwon, 2008) leading to a steady rise across Asia and worldwide (Stewart and Bhujel, 2007). As a consequence, the environmental problems caused by the intensive farming are a key constraint to its future growth (Tabthipwon, 2008).

The global aquaculture market shows a potential for development, but has still to deal with some problems, particularly in the context of health protection requirements, market instability and environmental impact (Focardi et al., 2005). These concerns arise because, as with any practice that interacts with the surrounding eco-systems, as the practice becomes more and more extensive, and increases in intensity on an international scale, so the environmental impact increases (Stewart and Bhujel, 2007). As a consequence of this expansion the use of chemicals, one of the most required external input for a successful production (Subasinghe, Barg et al. 1996), has become increasingly a part of the management, used mostly to treat diseased animals and to improve water quality.(Tonguthai 1996). Each chemical has specific effects and can be applied either singularly or in a combination (Tonguthai 1996).

It is obvious that chemicals and drugs play an important role in present aquaculture systems and in the development of Thai aquaculture. However the overuse, mainly of antibiotics, not only increases production costs but also intensifies adverse consequences (Tonguthai, 1996). Therefore it is necessary understanding the appropriate chemicals to use as a crucial part of good management (Yanong, 2003).

Dipterex and oxytetracycline are chemicals widely used in the treatment of freshwater fish. Their use in aquaculture for a long time may have resulted in severe environmental contamination and has generated concerns about their impact on public health (Tonguthai, 1996).

Dipterex is an organophosphate extensively used as a relevant treatment for various parasitic infestations (Ranzani-Paiva et al., 1997), representing an extremely important source of concern in commercial aquaculture (Guimarães et al., 2007). Most known studies are focused on the effect of organophosphate on the cholinesterase enzyme activities (Guimarães and Calil, 2008). In normal conditions of use, it is very quickly hydrolyzed to dichlorvos (2,2-dichlorovinyl dimethyl phosphate), which is much more toxic and a potential threat to non-targeted species such as fish, crabs, and shrimp (Guimarães et al., 2007). Dichlorvos blocks central ganglion cholinergic activity in invertebrates, which usually cause sicknesses in fishery and other forms of culture (Guimarães and Calil, 2008). Dipterex is highly toxic to many aquatic species like Daphnia, stoneflies, crayfish and several freshwater fish species (Anon., 2006). A 48h LC_{50} of 0.18 mg/L was reported for *Daphnia;* a 96h LC_{50} of 0.01 mg/L was reported for stoneflies, of 7.8 mg/L for crayfish, 1.4 mg/L for brook trout, 0.88 mg/L for channel catfish, 0.26 mg/L for bluegill (Hill and Camardese 1986) and 1.43mg/L for tilapia (Tuan 1992).

Oxytetracycline is an antibiotic widely used in aquaculture (Choo, 1994). It is a broad spectrum bacteriostatic antibiotic used in the treatment of a wide variety of infections, widely used as feed additive for therapy of systemic bacterial infections in farmed fish (Boleas et al., 2005). It is used to treat bacterial hemorrhagic septicemia and pseudomonas disease in catfish, to control ulcer disease, furunculosis, bacterial hemorrhagic septicemia and pseudomonas in salmonids (MacMillan et al., 2004). Some of the LC50 are >100 ppm/96 hr for *Lepomis macrochirus* (Bluegill) (Murphy and Peters, 1991b); 178 mg/L/96 hr for *Morone saxatilis* (Striped bass) (Vaituzis, 1988); >116 ppm/96 hr for *Oncorhynchus mykiss* (Rainbow trout) (Murphy and Peters, 1991a).

In this work several tests were performed involving the Nile tilapia larvae and the Zebrafish early-life stages exposed to dipterex and oxytetracycline in tropical conditions, to acquire a deeper knowledge of these chemicals effects and mechanisms of action on this organisms. Thus the principal objective of this work is to evaluate the toxicity of these chemicals in tropical environment.

2.2. Material and Methods

2.2.1. Nile tilapia experiments

2.2.1.1. Chemicals used

Dipterex/ Trichorfon ($C_4H_8Cl_3O_4P$), purity 100% (m/v), commercial product used in treatment practices in the Nam Sai Farms Co. Ltd.

Oxytetracycline hydrochloride ($C_{22}H_{24}N_20_9$ HCL), purity 50% (m/v), commercial product used in treatment practices in the Nam Sai Farms Co. Ltd.

2.2.1.2. Test organisms

The Nile tilapia larvae were selected after hatching from the Aquaculture and Aquatic Resources Management at Asian Institute of Technology AARM/ AIT hatchery ponds, the acute toxicity test lasted 96 hours before the yolk sac was completely absorbed and the fry are free-feeding.

2.2.1.3. Acute toxicity test

The experimental design was based on the OECD guideline on Fish, Early-life Stage Toxicity test (OECD, 1992) in static conditions, it consisted in three replicates/flasks per concentration filled with 100 ml of solution prepared using ASTM hard water (ASTM, 1998) and ten organisms.

The exposure conditions were the environmental conditions, i.e. 16:8 light: dark cycle, the temperature during the test was 26.5±0.5ºC and pH 7± 0.4. One hundred eighty animals were used per experimental trial.

For the oxytetracycline six treatments were used: control (ASTM) and oxytetracycline treatments at nominal concentrations of 150, 450, 1350, 4050 and 12500 mg/l. Test solutions were prepared by dilution of stock solution in ASTM hard water. This procedure was adopted for all assays realised. For the dipterex the used treatments were: control and dipterex treatments at nominal concentrations of 0.05, 0.2, 0.8, 3.2 and 12.8 mg/l.

Each experiment trial lasted 96 hours and the cumulative mortality was controlled every 24 hours, observing the immobility and/or absence of respiratory movement and/or white opaque colouration of central nervous system on larvae.

2.2.1.4. Statistical analysis

The 24h, 48h, 72h and 96h mortalities under exposure to dipterex and oxytetracycline were used to calculate the LC50 using the software package PriProbit (Throne et al., 1995).

2.2.2. Zebrafish experiments

2.2.2.1. Chemicals used

Dipterex/ Trichlorfon ($C_4H_8Cl_3O_4P$), purity 97 % (m/v) (HPLC), PESTANAL.product.

Oxytetracycline dihydrate ($C_{22}H_{24}N_2O_9.2H_2O$), purity 93.5% (m/v) (HPLC) SigmaUltra product.

2.2.2.2. Test organisms

Zebrafish from a culture established at the Department of Biology, University of Aveiro are maintained in carbon-filtered water at 28.0±2ºC under a 16:8 h light/dark photoperiod cycle. Conductivity is kept at 750±50 µS, pH at 7.5±0.5.

Adults are fed two times a day with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

2.2.2.3. Early-life stages assay

The assay was based on the OECD guideline on Fish Embryo Toxicity Test (OECD, 2006), in static conditions, and on the embryo test described by Fraysse et al. (2006).

Zebrafish eggs were collected approximately 30 min after natural mating, the coagulated eggs were discarded and the normal ones rinsed in water and checked under a stereomicroscope (Stereoscopic ZoomMicroscope—SMZ 1500, Nikon). Unfertilised eggs, with irregularities during cleavage or injured were discarded.

Three hundred and eighty four eggs were used, per experimental trial, and distributed in sixteen 24-well microplates. Eggs were placed individually in each well with 2.5ml of test solution.

In the oxytetracycline experiment eight treatments were used: control and oxytetracycline treatments at nominal concentrations of 10, 50, 100, 250, 500, 750 and 1000 mg/l (8 replicate each). Test solutions were prepared by diluting a stock solution in water from the culture system (Conductivity 750±50 µS and pH 7.5±0.5). The temperature during the test was 26.0±1ºC and the photoperiod was 16:8 h light/dark cycle. This procedure was adopted for all assays realised. For the dipterex the used treatments were: control and dipterex treatments at nominal concentrations of 2.5, 5.0, 10, 20, 40, 80, 160 mg/l.
Embryos and larvae were observed daily with the help of stereomicroscopy. (Magnification used for observations was x20, x30 and x40). Tests run for 5 days (120 hrs).

In the embryo phase, the following parameters were evaluated: egg coagulation, otolith and somite formation, eye and body pigmentation, tail detachment, pericardial oedema, yolk sac absorption delay, blood circulation, alteration on the amniotic liquid and hatching.

After hatching, the evaluated parameters were: oedemas, delay of the yolk sac absorption, tail deformation, blood circulation, eye deformation, undersize, posture and mortality.

2.2.2.4. Statistical analysis

Sigma Plot 10.0 statistical package (SPSS, Inc.) was used for statistical analyses. One-way ANOVA was performed; if significant results were found the Dunn's test was used to verify differences between tested concentrations and control.

Lethal concentrations at 50% (LC50) for early-life stages at 96 hrs and the Median Effective Concentration (EC50) for the evaluated parameters were calculated with the same statistical package.

2.3. Results

2.3.1. Dipterex and Oxytetracycline in Nile tilapia

Results obtained indicated a significant difference in the mortality of the larvae on the control treatment and the highest concentrations treatment in tested chemicals, dipterex and oxytetracycline. Dipterex showed acute toxicity to Nile tilapia larvae with a 96hr LC50 of 3.9612 mg/L (95% limits= 3.5433, 4.4893) (Figure 2.1), at the same conditions the LC50 (96 hr) obtained to the oxytetracycline was 1321.34 mg/L (Figure 2.2).

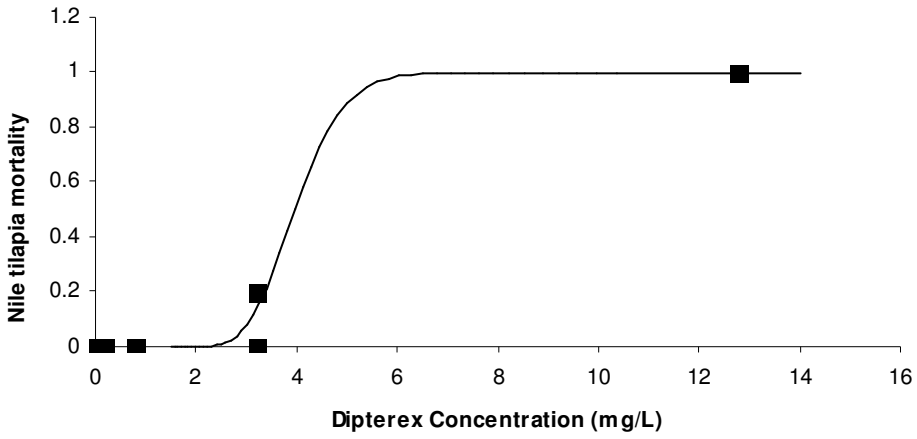

Figure 2.1 - Nile tilapia larvae mortality at 96 hr (Dipterex)

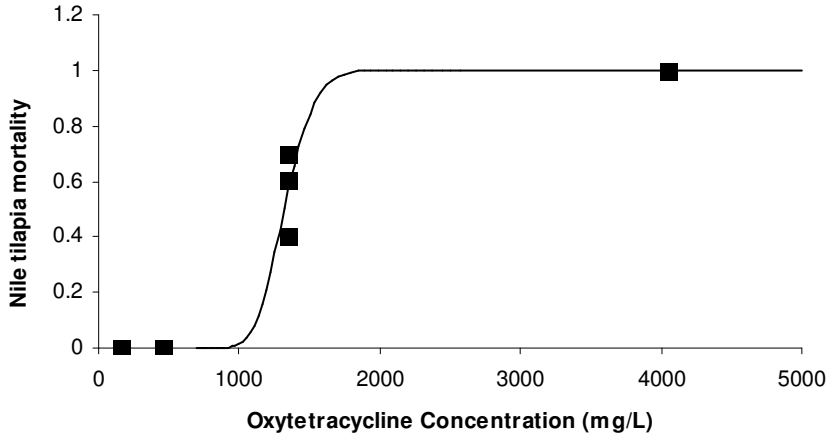

Figure 2.2 - Nile tilapia larvae mortality at 96 hr (Oxytetracycline)

2.3.2. Dipeterex in Zebrafish

Fertilized zebrafish eggs were exposed for 5 days (120 hr) to several concentrations of dipterex (Trichlorfon 500). Figure 2.3 shows the proportion of eggs and embryos that died during the experiment (black bars), the proportion of embryos that stayed alive (grey bars), that hatched (white bars) and finally proportion of larvae that died (dark grey). The dipterex showed acute toxicity for

D. rerio embryos and larvae with a LC50 (96 hr) of 25.4±4.37 mg/L (mean±95% CI; Figure 2.4).

In the Day 2 (first 48 hr) 89.6% of the embryos exposed to 160mg/L died, at the Day 3 (72 hr) the remaining embryos of the 160 mg/L treatment and the embryos exposed to 80 mg/L died as well; the next day (96 hr) 97.9% of the embryos in 40 mg/L concentration died and the survivors in this concentration died the next day (Figure 2.4).

At the Day 2 some embryos of the control and 2.5 and 5 mg/L concentrations started hatching but in general the embryos hatched until the Day 3 (72 hr) (Figure 2.5). In the 40 mg/L treatment only 23.75% had hatched at this day showing a delay from the control treatment (Kruskal–Wallis *H=28.029 p< 0.001*), at Day 4 (96 hr) 97.9% of the embryos and larvae died and the remaining group hatched but died the next day (120 hr) (see Figure 2.4 and Figure 2.5).

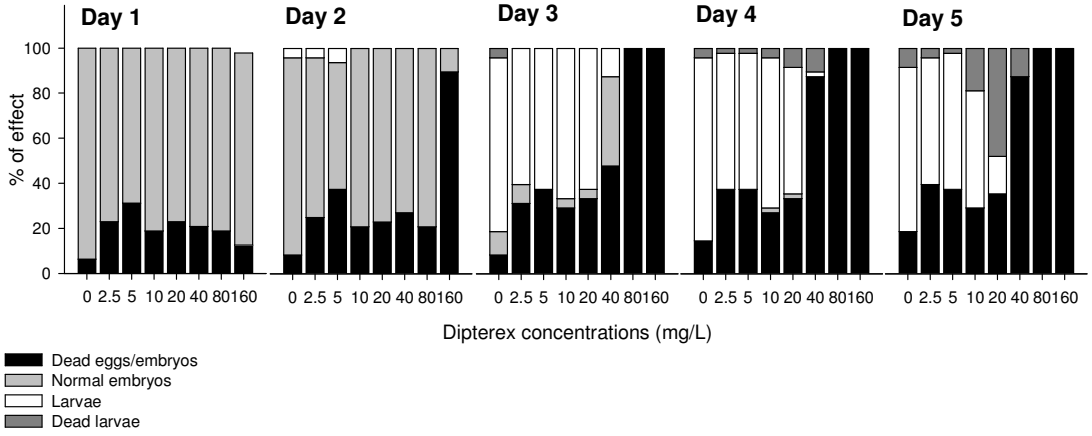

Figure 2.3 - General overview of Dipterex effects on *Danio rerio* embryo and larvae during the 5 days (120 hr) of exposure.

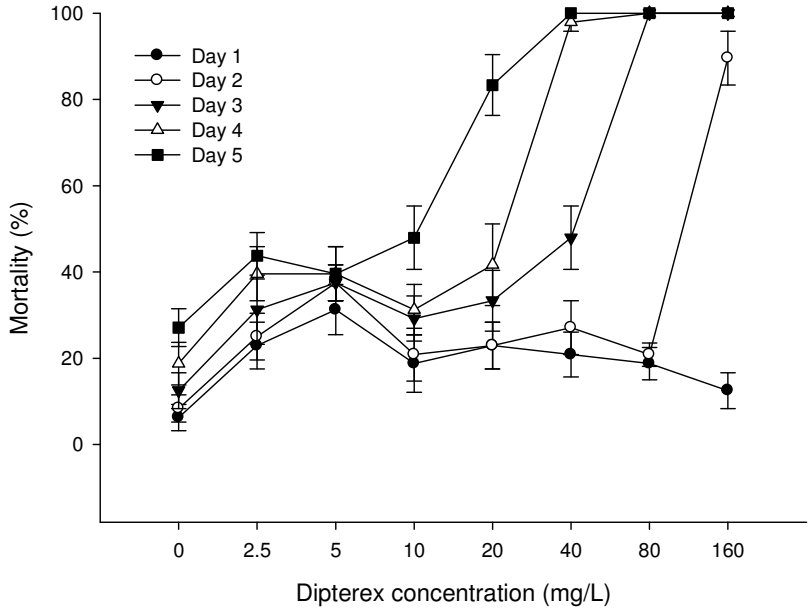

Figure 2.4 - Mortality of embryos and adults during the trial

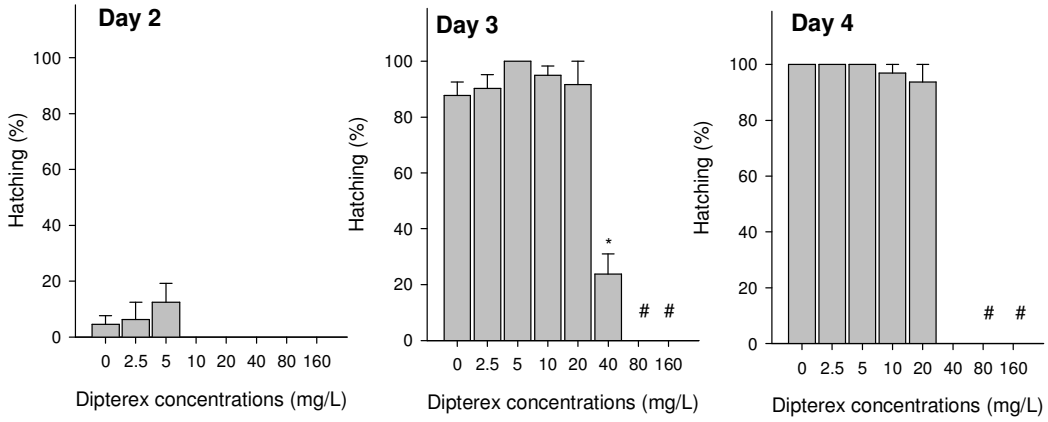

Figure 2.5 - Hatching of *D. rerio* embryos during the exposure (# all animals of the treatment died, * mean significantly different from control treatment (Dunn's Method P< 0.05))

Developmental parameters proved to be excellent indicators of dipterex toxicity. Table 2.I summarises effects on the analysed parameters.

At the Day 1 embryos development in the control, 2.5 and 5 mg/L treatments was normal, as described by Kimmel et al. (1995). But at the 160 mg/L treatment a statistical significance was observed on the eye and body

pigmentation (Kruskal–Wallis *H= 62.681; p< 0.001*) and on the tail detachment (Kruskal–Wallis *H= 39.401; p< 0.001*) (Table 2.I, Figure 2.6 and Figure 2.7).

After 48 hr, Day 2, statistical significant differences were observed at a concentration of 80 and 160 mg/L on the body pigmentation (Kruskal–Wallis *H= 48.405; p< 0.001*), delay of the yolk sac absorption (Kruskal–Wallis *H= 43.792; p<0.001*) and at 80mg/L on the pericardial oedema (Kruskal–Wallis *H= 41.508; p<0.001*) (Figure 2.8 and Figure 2.9).

At Day 3 significant differences were observed in the larvae at the 20 and 40 mg/L treatments in the delay of the yolk sac absorption (Kruskal–Wallis *H= 36.557; p<0.001*) and in the frequency of pericardial oedema (Kruskal–Wallis *H= 32.127; p< 0.001*) (Figure 2.10 and Figure 2.11).

At Day 4 (96 hr) the results were similar, with a statistical significance in the concentration 20 mg/L, showing a delay on the yolk sac absorption (Kruskal–Wallis *H= 26.651; p<0.001*) and pericardial oedema (Kruskal–Wallis *H= 23.487; p< 0.001*) (Figure 2.12 and Figure 2.13).

At the last day of the larvae exposure, Day 5, the 20 mg/L treatment continue to show the deficient absorption of the yolk sac (one-way ANOVA: $F_{4,35}=15.49$; $p< 0.001$) and pericardial oedema (Kruskal–Wallis H= 13.068; p= 0.011) (Figure 2.14).

Dipterex is teratogenic at concentrations higher then 5 mg/L leading to some development delay, significant effects on the mortality and hatching.

These results showed the influence of the dipterex on the embryos development causing effects at several levels.

Table 2.I - Effects of different concentrations of Dipterex on developmental parameters of zebrafish embryos and larvae.

	D1	D2	D3	D4	D5
Eye pigmentation	LOEC= 160	LOEC= 160	–	–	–
Body pigmentation	LOEC= 160	**56.580** (3.388)	–	–	–
Somite/ Otolith formation	n.e.	–	–	–	–
Tail detachment	DR	–	–	–	–
Pericardial oedema	LOEC= 160	**42.160** (4.261)	**12.250** (29.030)	**11.860** (2.244)	DR
Yolk sac absorption	n.e.	**61.760** (3.113)	**13.660** (1.110)	**7.425** (1.683)	**24.200** (49.670)
Alteration on the amniotic liquid	n.e.	n.e.	–	–	–
Tail deformation	–	**65.810** (3,880)	DR	**10.560** (1.917)	DR
Tail blood circulation	–	n.e.	n.e.	n.e.	n.e.
Eye deformation	–	n.e.	n.e.	n.e.	n.e.
Undersize	–	–	–	**10.660** (2.924)	DR
Posture	–	–	–	LOEC= 20	–

Bold values are EC_{50} (in mg/L) of dose responsive endpoints followed by the Standard Error between brackets.
"n.e." means no effect on the endpoint analysed.
LOEC (lowest observed effect concentration) is presented.
DR: dose responsive Endpoint (however no EC50 value could be calculated)

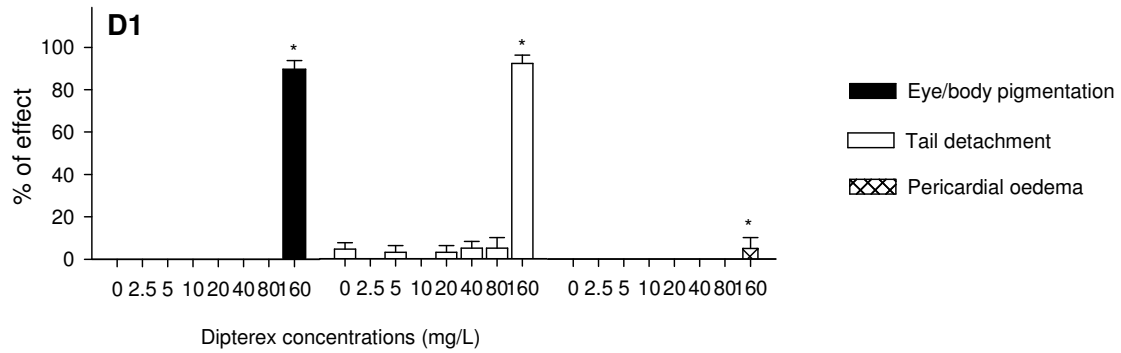

Figure 2.6 - Endpoints incidence and frequency at different concentrations at Day 1. * mean significantly different from control treatment (Dunn's Method P< 0.05)

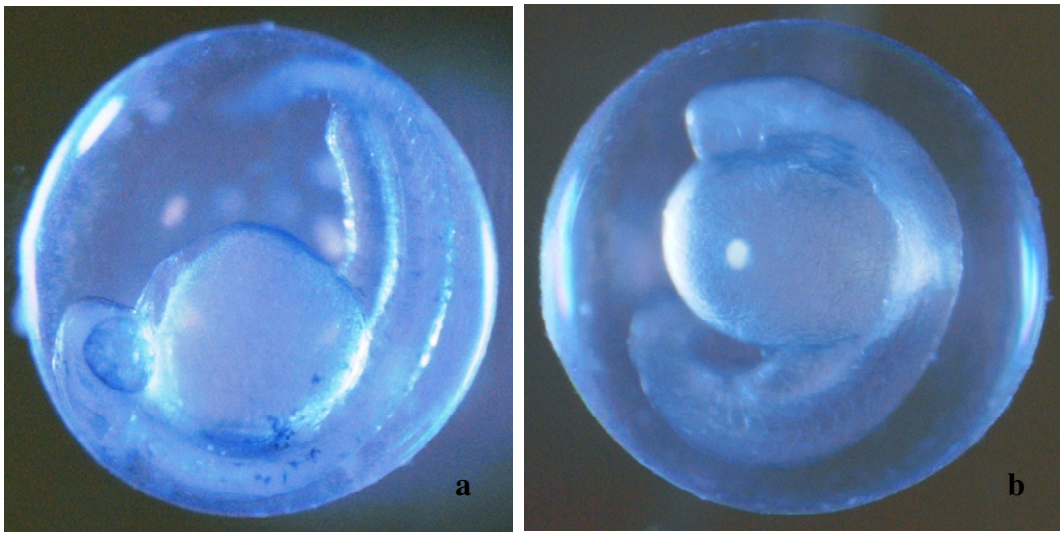

Figure 2.7 - Zebrafish embryos exposed to Dipterex at Day 1 (24 hr): a- control, normal embryo with adequate body and eye pigmentation (x 40); b- embryos exposed to 160 mg/L with weakly pigmentation retina and body and abnormal tail detachment (x40)

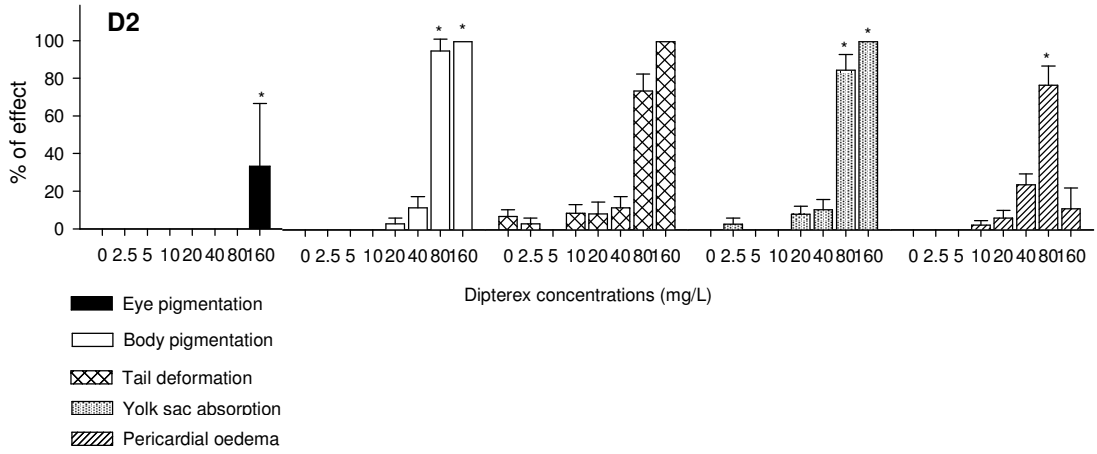

Figure 2.8 - Endpoints incidence and frequency at different concentrations at Day 2. *mean significantly different from control treatment (Dunn's Method P< 0.05)

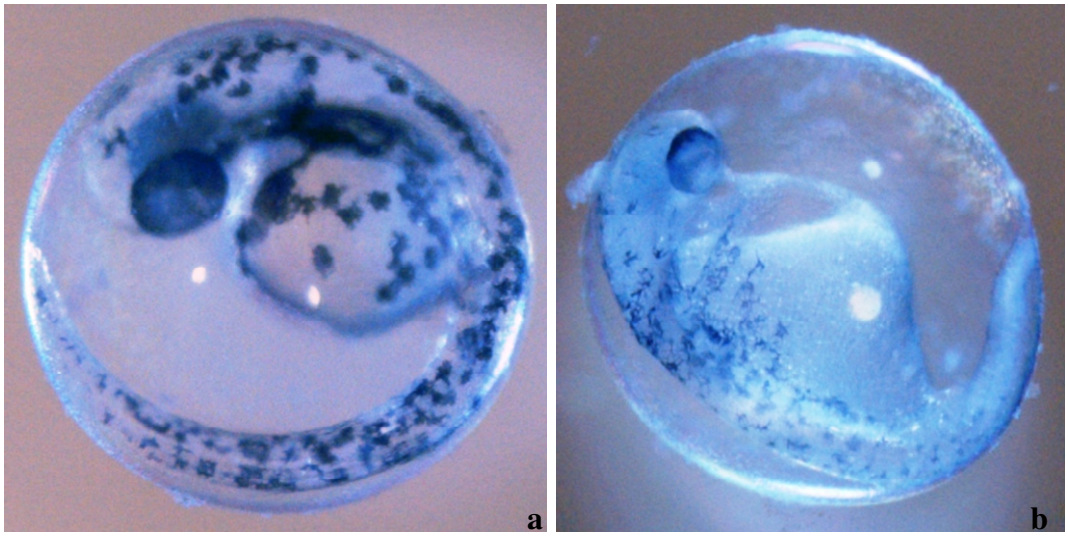

Figure 2.9 - Zebrafish embryos exposed to Dipterex at Day 2 (48hr): a- control, normal embryo development (x30); b- embryos exposed to 80 mg/L with pericardial oedema, weakly body pigmentation and yolk sac absorption delay (x30)

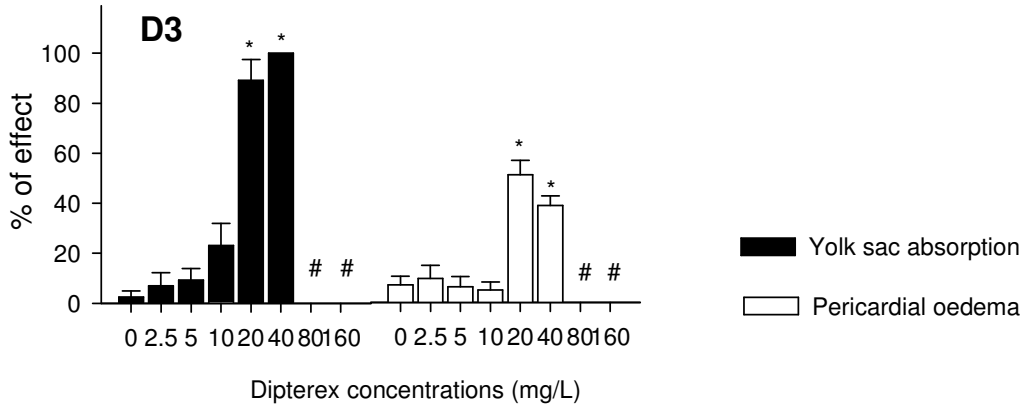

Figure 2.10 - Endpoints incidence and frequency at different concentrations at Day 3. # all animals of the treatment died, * mean significantly different from control treatment (Dunn's Method P< 0.05)

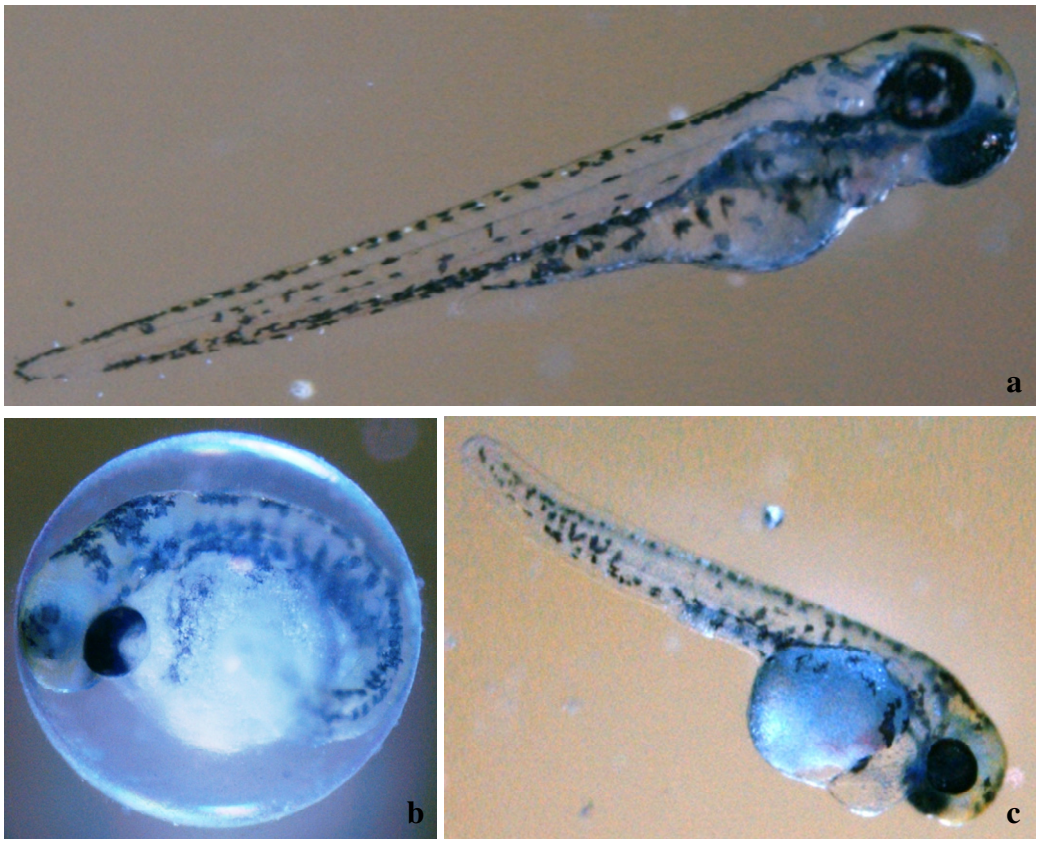

Figure 2.11 - Zebrafish embryos and larvae exposed to Dipterex at Day 3 (72 hr): a- larvae with normal with well-developed tail and normal body structure (x20); b- embryo exposed to 40 mg/L with hatching delay (x40); c- larvae exposed to 40 mg/L with pericardial and yolk sac absorption delay (x20)

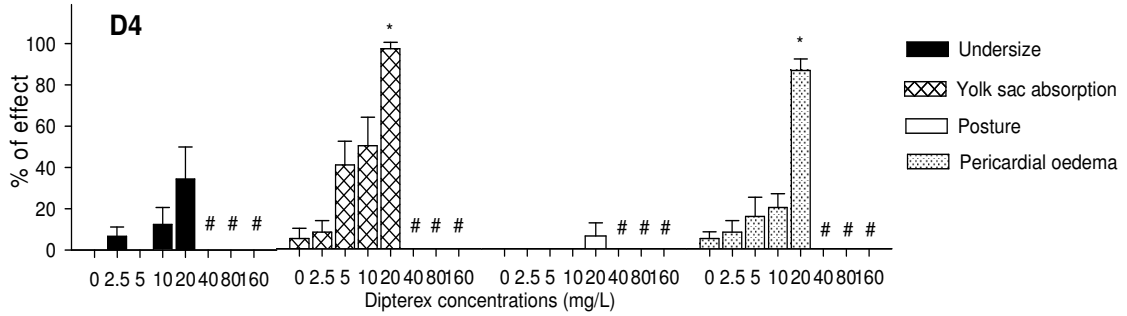

Figure 2.12 - Endpoints incidence and frequency at different concentrations at Day 4. # all animals of the treatment died, * mean significantly different from control treatment (Dunn's Method P< 0.05)

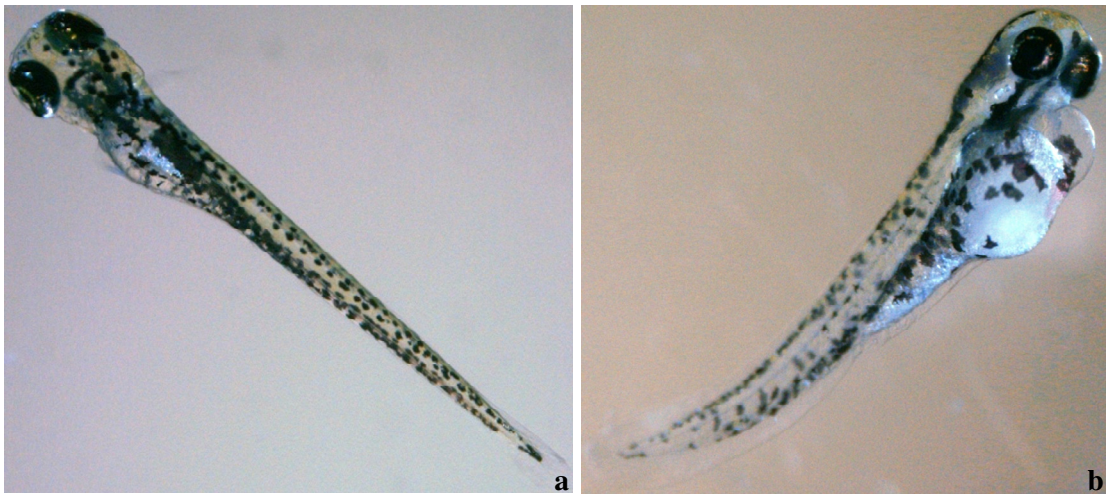

Figure 2.13 - Zebrafish embryos exposed to Dipterex at Day 4 (96 hr): a- normal larvae with normal body structure (x20); b- larvae exposed to 20 mg/L with yolk sac absorption delay, tail deformation and pericardial oedema (x30)

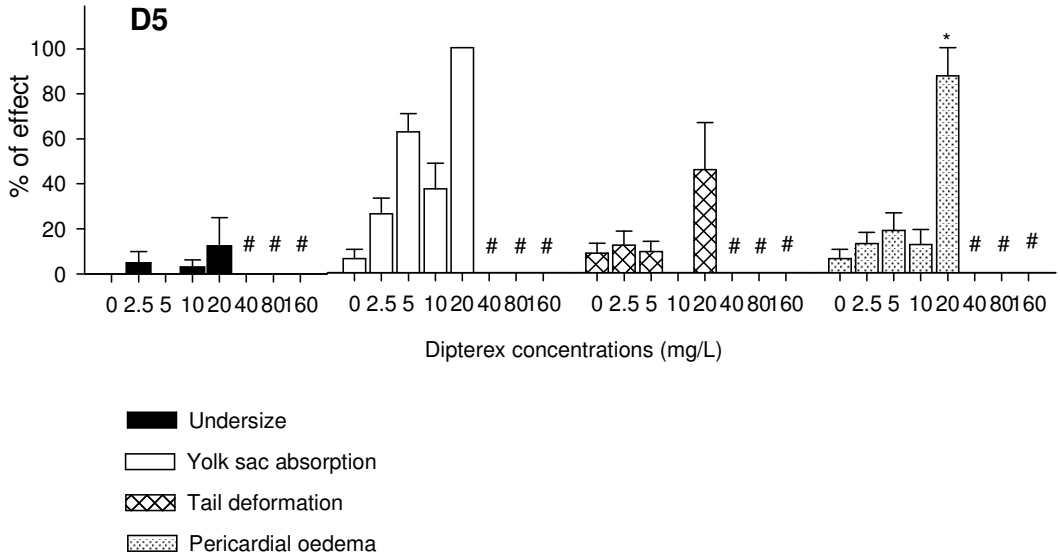

Figure 2.14 - Endpoints incidence and frequency at different concentrations at Day 5. # all animals of the treatment died, * mean significantly different from control treatment (Dunn's Method P< 0.05)

2.3.3. Preliminary tests with Oxytetracycline in Zebrafish

Preliminary tests were performed under the same experimental conditions using concentrations of 0, 10, 50, 100, 250, 500, 750 and 1000 mg/L of oxytetracycline dihydrate, but the chemical was not soluble at concentrations higher than 250 mg/L and deposited and accumulated in the bottom of each well, so the results are not conclusive.

2.4. Discussion

The chemicals tested in this work (dipterex and oxytetracycline) are widely used in aquaculture practices in tropical environment; this use can affect various aquatic organisms and the surrounding environment through unexpected modes of action.

2.4.1. Dipterex and Oxytetracycline in Nile tilapia

The preliminary acute toxicity tests made with dipterex and oxyteracycline on Nile tilapia are not conclusive; more tests should be made to obtain the real LC50, similar to the LC50 determined on previous studies with different fish species.

However dipterex showed to be toxic to Nile tilapia causing larvae mortality with a LC50 (96 hr) of 3.96 mg/L similar to the LC50 (96hr) of 2.5 mg/L found for brook trout, (Hill and Camardese, 1986) and of 1.43mg/L for tilapia (Tuan, 1992).

The LC50 (96hr) obtained to the Oxytetracycline was 1321.34 mg/L, higher than the 100 mg/L found in previous works for bluegill (Murphy and Peters, 1991b), striped bass (Vaituzis, 1988) and rainbow trout (Murphy and Peters, 1991a), for this reason oxytetracycline hydrochloride can be classify as practically non-toxic (Vaituzis, 1988) to Nile tilapia in tropical environment. This agrees with reports from EPA (1993) concluding, based on several acute toxicity studies published in literature, that oxytetracycline is practically non-toxic to fish and aquatic invertebrates.

In a work testing the several levels of pharmaceuticals in river basins (Schowanek and Webb, 2000), the oxytetracycline Predicted No- Effect Concentration (PNEC) to organisms in ecosystems obtained was $2.3x10^{-4}$ mg/L much lower then the LC50 obtained, confirming it is practically non- toxic to Nile tilapia.

This study is important as no previous work tested the effect of oxytetracycline in tropical conditions while it is one of the most used antibiotics in South East Asia aquaculture practices.

2.4.2. Dipterex in Zebrafish

Zebrafish early- life stages test was very informative about the dipterex toxicity. The normal embryo development described by Kimmel et al. (1995) was affected in some tested concentrations.

Embryos mortality was dependent on the dipterex concentration and a LC50 (96 hr) of 25.4 mg/L was calculated. The LC50 (96 hr) of Dipterex to rainbow trout, brook trout and catfish is 1.4, 2.5, 0.88 mg/L respectively (Hill and Camardese, 1986) tested in fry and adult fish, much lower than the LC50 (96 hr) obtained in this work. This can be explained by the different test conditions as the toxicity can be affected by many factors including temperature, pH and water hardness, which may have different effects across species (Hill and Camardese, 1986). Or, as it was proved in some works, the chorion of the egg may act as a barrier of embryo protection for some toxicants (Oliveira et al., 2009) as the potassium dichromate tested in common carp (Krejčí and Palíková, 2006). In this work, egg chorion may also have protected the embryo against dipterex, decreasing its sensitivity when compared with other fish life stages.

Relatively to hatching time, used in this work as an endpoint, a significant delay was observed on embryos exposed to 40 mg/L at Day 3, it was verify that these embryos never hatched and died at Day 4. No works were found in literature to support the hatching delay and the teratogenic responses to dipterex during embryo development in *D. rerio*.

The mechanisms of embryotoxicity of dipterex are not yet described in aquatic organisms. The tail deformation or spine deformation observed in some larvae (Figure 2.13) can result from the depletion or deregulation of ions like phosphorous and calcium or with a reduction in myosin and myotonia required for normal development (Cheng et al., 2000). The weakly body pigmentation (Figure 2.7) was reported in other works with Zebrafish as a response to exposition to copper (Cu), cadmium (Cd) (Nguyen and Janssen, 2002) and lead (Pb) (Ozoh, 1980). In this case the most frequent effect was the incidence of pericardial oedema (Figure 2.9, Figure 2.11 and Figure 2.13), regularly associated with leaks in the endothelial vessels and usually results in cardio-vascular dysfunctions (Hallare et al., 2004).

Early life-stages assay contributed with relevant information regarding anomalies in embryo and larvae development, showing that dipterex is teratogenic at concentrations higher then 5 mg/L, leading to some development delay and consequent death.

The large difference of the dipterex LC50 (96 hr) values between the Nile tilapia (3.96 mg/L) and Zebrafish (25.41 mg/L) can result, besides of the different characteristics of the species, from the life stages when organisms were tested, as described above, the chorion of the egg may act as a barrier of embryo protection for some toxicants (Krejčí and Palíková, 2006). Some authors (Nguyen and Janssen, 2002) stated that some chemicals have low embryotoxicity to African catfish (*Clarias gariepinus*) eggs comparatively to post-hatched embryos (larvae), showing higher sensitivity in larvae stage than in embryo stage. Thereby the Nile tilapia larvae used in the preliminary tests showed higher sensitivity to dipterex than the Zebrafish embryos.

However, both values are among the LC50 estimates from 0.23 mg/L for bluegill sunfish to 110 mg/L for fathead minnow (EPA, 1997); these values show that the acute toxicity of dipterex ranges from highly toxic to practically non-toxic to freshwater fish (EPA, 1997).

The EEC (Estimate Environmental Concentration) at 96 hr determined for freshwater fish (EPA, 1997) was 0.224 mg/L, according to this work the accumulation has no teratogenic effects as the dipterex was not harmful from the control to the 5 mg/L treatment, but more data are required in order to confirm the risk of its use.

Although this work indicates that dipterex has low levels of toxicity in the case of Zebrafish, this kind of studies are important as no adequate data about the dipterex effects exist for tropical environment and it is extensively used in South East Asia aquaculture.

It is important to continue the study about the toxic effects of these chemicals widely used in aquaculture practices and the organism's responses in tropical conditions as the toxicity can greatly differ according to the environmental factors, as pH and temperature. They should also be tested in mixture with other chemicals since the levels of toxicity can change such as the effects to the environment.

2.5. References

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3. Conclusions and Final remarks

As stated before, the development of Aquaculture in Thailand, a tropical environment, over the past 20 years quickly provided an effective source of protein playing an important role in the national economy. As advances in knowledge of aquaculture practice slowly began to gather pace, the rise in production predictably gathered pace (Stewart and Bhujel, 2007). This increment led to the use of a wide variety of chemicals and biological products to treat fish, water and sediment of ponds (Tonguthai, 1996) in semi-intensive and intensive farming. This increase in the use of chemicals resulted in increasing environmental concerns such as the effects and fates of the chemicals and their residues in the water and sediment, natural aquatic communities and microorganisms (Subasinghe et al., 1996).

Dipterex and oxytetracycline were the focus of this study as they are two of the chemicals that farmers use as insecticide in treatment baths and antibiotic mixed with food, respectively (Tonguthai, 1996). Thus, it is essential to know their effects in water, soil, farmed animals and surrounding environment in general.

The results of the preliminary test with oxytetracycline showed that apparently it does not constitute a risk to the environment, at the concentrations they are used, as it was practically non-toxic to Nile tilapia with a LC50 (96 hr) of 1321.34 mg/L moreover, the test with Zebrafish showed no lethal toxicity for the concentrations tested (the chemical was not soluble at concentrations higher than 250 mg/L and deposited and accumulated at the bottom of each well) making the results not reliable.

Dipterex was more toxic to Nile tilapia than to Zebrafish. This fact can be explained by the difference in the life stages of the two species tested, the Zebrafish was tested in embryo stage and the chorion of the egg may act as a barrier protecting the embryo from some toxicants. The Nile tilapia LC50 (96 hr) of 3.96 mg/L is not a definitive value as it was obtained in a preliminary test. Dipterex, although less toxic to Zebrafish with a LC 50 (96 hr) of 25.41 mg/L, caused hatching delay at 40 mg/L and embryo development anomalies.

Although the chemicals were not very toxic to the freshwater fish species, this work was important because no adequate data and information existed about the action and toxicity of these chemicals in tropical environments like Thailand. Aquaculture is currently a rising practice and the consequent high productivity plays an important role in the socio-economic development (Tabthipwon, 2008), due to this fact chemicals use is increasing as a part of management to achieve a quick and efficient production (Tonguthai, 1996) and this increment should be accomplished with convenient evaluation of impact to ecosystems. However, the control of the adverse impacts should go further; the toxicity tests are fundamental, but an investment should be done on the monitoring of the quantities and quality of the chemicals used, the treatment of waste products and the discharges of effluents. Some aquaculture farms in Thailand utilize chemicals in higher concentrations than the necessary and use products identified by their trade names but with no further information on the ingredients.

It is also ecologically important to test these chemicals in combination to other chemicals applied in the same practice, as they can be no harmful when used individually but dangerous when mixture with other chemicals.

Therefore, it is important to assess as well as the adverse environmental impacts of these chemicals, used in aquaculture systems in the tropics, to promote better management practices, thus reducing their use and, consequently, their impacts to the environment.

3.1. References

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